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Stereoselectivity and Enantiomer-Enantiomer Interactions in the Binding of Ibuprofen to Human Serum Albumin

TOMOO ITOH,* YOSHIKAZU SAURA, YASUYUKI TSUDA, AND HIDEO YAMADA
Department of Pharmacokinetics and Biopharmaceutics, School of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo, Japan

ABSTRACT Binding of ibuprofen (IB) enantiomers to human serum albumin (HSA) was studied using a chiral fluorescent derivatizing reagent, which enabled the measurement of IB enantiomers at a concentration as low as 5×10^{-8} M. Scatchard analyses revealed that there were two classes of binding sites for both enantiomers. For the high affinity site, the number of the binding sites was one for both enantiomers, and the binding constant of R-IB was 2.3-fold greater than that of S-IB. The difference in the affinity at the high affinity site may result in the stereoselective binding of IB enantiomers at therapeutic concentrations. It was confirmed that the high affinity site of IB enantiomers is Site II (diazepam binding site) by using site marker ligands. Also, significant enantiomer-enantiomer interactions were observed in the binding. The binding data were quantitatively analyzed and a binding model with an assumption of competitive interactions only at the high affinity site simulated the binding characteristics of IB enantiomers fairly well. *Chirality 9:643–649, 1997.*

KEY WORDS: ibuprofen; stereoselective; binding; HSA; interaction; fluorescent

Ibuprofen (Fig. 1) is a nonsteroidal anti-inflammatory drug that possesses a chiral center in the propionic acid moiety. It has been used clinically as the racemate, although the pharmacological activity resides mainly in the S-enantiomer.[1] Differences in pharmacokinetic properties between the enantiomers have been reported, including the unidirectional conversion from R-IB to S-IB.[2–5]

It has been reported that plasma protein binding is stereoselective for many chiral drugs,[6–9] including IB[10–15] and other 2-arylpropionic acid derivatives.[11,16–19] Interactions between the enantiomers in the binding are also suggested.[10,14,15,17,18] Although there are several reports on the stereoselectivity and enantiomer-enantiomer interactions in the binding of IB in plasma or HSA solution,[13–15] some discrepancies exist between these reports. One of the problems in accurately measuring the binding of IB is the sensitivity of the assay method. Since IB is an extremely highly bound drug, its unbound concentration in plasma is very low. Without an assay method of high sensitivity and stereospecificity, it is impossible to measure very low concentrations of unbound IB enantiomers, which in turn will result in unreliable binding parameters.

In the present study, IB enantiomers were derivatized to fluorescent diastereomers with a chiral fluorescent derivatizing reagent that reacts with carboxylic acid groups.[20] The derivatized diastereomers were analyzed with HPLC using an achiral stationary phase. Enantiomer-enantiomer interactions as well as stereoselectivity in the binding of IB enantiomers to HSA were studied in detail using the present analytical method.

MATERIALS AND METHODS

HSA used in the present study was purchased from Sigma Chemical Co. (St. Louis, MO). It was free from fatty acid and globulin (product number: A-3782), and was used without further purification. Racemic ibuprofen (rac-IB), S-(+)-4-nitro-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole (S-(+)-NBD-APy) and triphenylphosphine were purchased from Tokyo Kasei Industries (Tokyo, Japan). R-IB and S-IB were purchased from Research Biochemicals Inc. (Natick, MA). The enantiomeric purity of each enantiomer was measured with HPLC using a chiral stationary phase (Sumichiral OA-2500, Sumika Chemical Analysis Service, Osaka, Japan) and was greater than 98.8% for both enantiomers. 2,2′-Dipyridyl disulfide and diazepam were purchased from Wako Pure Chemicals (Osaka, Japan). Phenylbutazone, digitoxin, and naproxen were purchased from Sigma Chemical Co. All other reagents used in the present study were either analytical or HPLC grade.

Protein Binding Study

HSA was dissolved in pH 7.4 isotonic phosphate buffer (a mixture of 0.067M disodium hydrogen phosphate and 0.067M sodium dihydrogen phosphate with the isotonicity adjusted with NaCl), and the binding of IB was measured

*Correspondence to: Tomoo Itoh, Dept. of Pharmacokinetics and Biopharmaceutics, School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo, Japan. E-mail: itoht@pharm.kitasato-u.ac.jp.
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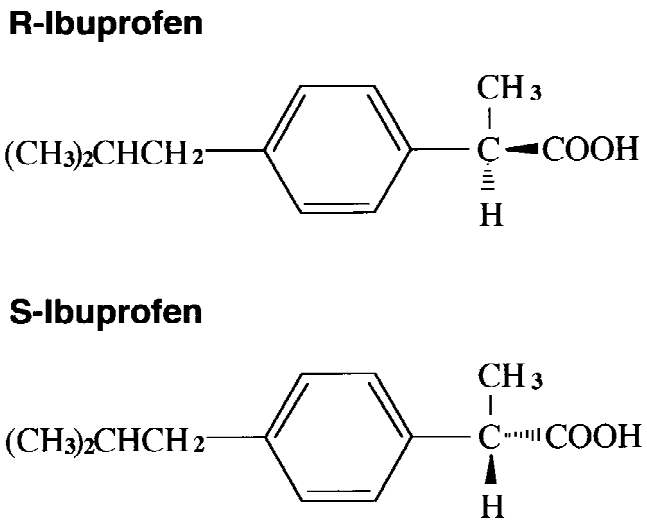

Fig. 1. Chemical structures of ibuprofen (IB).

in 0.4% (6×10^{-5} M) HSA solution at 37°C by an ultrafiltration method. An Amicon Centrifree® device was used for ultrafiltration with a type YMT-3 membrane (Amicon Division, W.R. Grace & Co., Beverly, MA).

Two milliliters of HSA solution was pre-incubated for 5 min at 37°C, a 10-µl aliquot of IB solution (rac-IB, R-IB, or S-IB dissolved in ethanol at an appropriate concentration) was added, and the mixture was vortexed. A 0.1-ml aliquot of the mixture was used for subsequent derivatization (see below) to determine the total (bound plus unbound) concentration of IB. A 1-ml aliquot of the mixture was transferred to an ultrafiltration device, and centrifuged at 1,000*g* for 1 min at 37°C. The ultrafiltrate obtained (approximately 150 µl) was used for derivatization (see below) to determine the unbound concentration of each enantiomer. It was confirmed that no degradation or adsorption to the device occurred under these experimental conditions. The concentration of ethanol in HSA solution was 0.25–0.5%. It was confirmed in the preliminary study that ethanol in this concentration range does not affect the binding of IB to HSA.

In order to study the effects of site marker ligands on the binding of IB, a 5-µl aliquot of IB solution and 5 µl of ethanol solution containing an appropriate concentration of a site marker ligand was added to 2 ml of HSA solution. Binding of IB was measured as described above. As a control, 5 µl of ethanol without site marker ligands was added to HSA, and the binding was measured. Site marker ligands were phenylbutazone for Site I, diazepam for Site II, and digitoxin for Site III. The concentration of site marker ligands was 3.0×10^{-4} M in HSA solution.

Sample Preparation

A 0.1-ml aliquot of the HSA solution or the ultrafiltrate was mixed with 1 ml of 0.2 M phosphate buffer (pH 2.0) and 50 µl of naproxen (100 µg/ml in ethanol) was added as an internal standard. Ether (5 ml) was added and the mixture was vortexed. The mixture was centrifuged, and the ether layer was partially evaporated with a rotary evaporator under vacuum at 30°C. The aqueous layer was extracted with 5 ml of ether for the second time, and the ether layer obtained was added to that previously obtained. The ether layers were vortexed with 2 ml of 0.01 M phosphate buffer (pH 6.0) for a clean-up. The mixture was centrifuged, and the ether was evaporated to dryness. Acetonitrile (0.1 ml) was added to the residue and the mixture was vortexed. This acetonitrile solution was subsequently used for the reaction with a fluorescent derivatizing reagent as described by Toyo'oka et al.[20] Briefly, 100 µl of 10 mM S-(+)-NBD-APy, 150 µl of 10 mM 2,2′-dipyridyl disulfide, and 150 µl of 10 mM triphenylphosphine were added to the 0.1 ml acetonitrile solution. All these reagents were dissolved in acetonitrile. The mixture was left to stand at room temperature for 4 h, and 20 µl aliquot was injected on to the HPLC column.

HPLC Conditions

A high-performance liquid chromatographic assay was used to determine the concentrations of IB enantiomers. The HPLC system consisted of a dual-piston pump, a fluorescent detector, an auto-injector, and an integrator. The analytical column was Cosmosil® ($5C_{18}$-AR, 4.6 mm [i.d.] × 250 mm; Nakalai Tesque Co., Kyoto, Japan). Fluorescent derivatives were analyzed with a solvent gradient method with the mobile phase composition (acetonitrile/water) being varied between 40/60 and 100/0. The flow rate was 1 ml/min. Fluorescent derivatives were detected with the excitation and emission wavelengths of 470 and 540 nm, respectively.

Data Analysis

Binding parameters were calculated according to the Langmuir equation (Eq. 1) using a non-linear least squares method (MULTI):[21]

$$r = \sum_{i=1}^{N} \frac{n_i \cdot K_i \cdot C_f}{1 + K_i \cdot C_f} \quad (1)$$

where r is the number of bound drug molecules per albumin molecule, n_i is the number of binding sites for the ith site, K_i is the binding constant for the ith site, and C_f is the unbound drug concentration.

RESULTS

Three peaks corresponding to R-IB, S-IB, and naproxen were separated on the baseline of the HPLC chromatogram. There were no peaks interfering with any of the three peaks on the chromatogram of the blank samples (data not shown). Calibration curves were linear for the HSA solution and ultrafiltrate over the concentration range between 0–100 µg/ml (0–4.9×10^{-4} M) with the correlation coefficients being greater than 0.999 for both R-IB and S-IB. The detection limit was 0.01 µg/ml (4.9×10^{-8} M).

In order to clarify the possibility of racemization during the present analytical procedure, the enantiomeric purity of IB was compared before and after the derivatization. No enantiomerization from S-IB to R-IB was observed, whereas a very small extent (1–2%) of enantiomerization from R-IB to S-IB was observed.

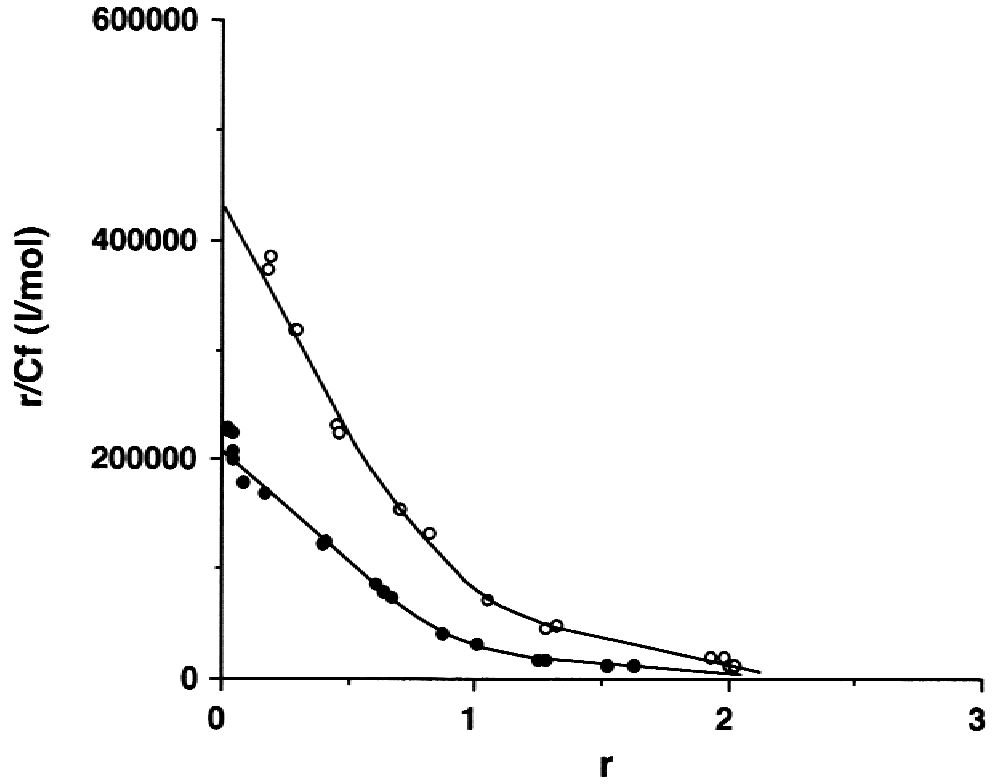

Fig. 2. Scatchard plots for the binding of IB enantiomers in 0.4% HSA. The binding of each enantiomer was measured in the absence of the antipode. ○: R-IB, ●: S-IB, —: simulated curves using the parameter values in Table 1.

Binding of IB enantiomers was measured individually using R-IB and S-IB. The unbound fraction of S-IB was greater than that of R-IB, indicating a difference in the binding of IB enantiomers to HSA. Binding data were analyzed with Scatchard plots, which are shown in Figure 2. Both enantiomers showed two classes of binding sites, and the binding parameters calculated according to Eq. 1 are listed in Table 1. When the binding parameters of the two enantiomers were compared, it was revealed that the high affinity binding site is responsible for the stereoselective binding of IB enantiomers to HSA. The binding constant of R-IB at the high affinity site was 2.3-fold greater than that of S-IB.

The effects of various site marker ligands on the binding of IB enantiomers were examined in order to identify the binding sites. Binding of R-IB and S-IB was measured in the absence or presence of each site marker ligand at a concentration of 3×10^{-4} M. The concentration of each enantiomer was 2.5×10^{-5} M. The results are shown in Figure 3A and B for R-IB and S-IB, respectively. Both R-IB and S-IB were displaced from the binding site(s) in the presence of diazepam, suggesting that both enantiomers may bind to Site II. In contrast, phenylbutazone and digitoxin did not affect the binding of either R-IB or S-IB, indicating that Site I or III is not involved in the binding of IB enantiomers. Moreover, S-IB was displaced by diazepam to a greater extent than R-IB, which was in agreement with the greater binding affinity of R-IB at the high affinity site.

In order to study enantiomer-enantiomer interactions, the binding of each enantiomer was examined in the presence of various concentrations of the antipode. The unbound fraction of R-IB (Fu(R)) was measured at a constant concentration (2.5×10^{-5} M) of R-IB in the presence of various concentrations (0–2.5×10^{-4} M) of S-IB; the R/S ratio varied from 1/0 to 1/10. On the other hand, the unbound fraction of S-IB (Fu(S)) was measured at a constant concentration (2.5×10^{-5} M) of S-IB with various concentrations (0–2.5×10^{-4} M) of R-IB. Both Fu(R) and Fu(S) increased significantly when the concentration of the antipode was increased (Fig. 4A and B), suggesting that IB enantiomers displace each other at the common binding site.

Since the high affinity site of both enantiomers appears to be Site II (Fig. 3A and B) and significant interactions were observed between the enantiomers (Fig. 4A and B), various models with competitive interactions were tested to account for the enantiomer-enantiomer interaction data shown in Figure 4A and B. In search of the best fit model, the numbers of the binding sites were fixed to integer numbers. The n_1 values for the high affinity sites were assumed to be one for both enantiomers. The n_2 values for the low affinity sites were assumed to be two for R-IB and one for S-IB since the n_2 values of R-IB and S-IB were close to two and one, respectively (Table 1). When the numbers of the binding sites were fixed to integer numbers, the binding constants obtained from the analysis of the binding data (Fig. 2) with a Langmuir equation (Eq. 1) were 4.1×10^5, 2.0×10^5, 8.3×10^3, and 4.8×10^3 M^{-1} for $K_1(R)$, $K_1(S)$,

TABLE 1. Binding parameters of R-IB and S-IB

Reference		n_1[a]	K_1 (M^{-1})[b]	n_2[a]	K_2 (M^{-1})[b]
This study	R-IB	0.95	4.2×10^5	1.8	1.2×10^4
	S-IB	1.0	1.8×10^5	1.2	6.5×10^3
Rahman et al.[13]	R-IB	1.4	3×10^6	4.7	1.9×10^5
	S-IB	1.2	2×10^6	4.5	1.8×10^5
Paliwal et al.[14]	R-IB	1	5.75×10^5	—[c]	—[c]
	S-IB	1	2.10×10^5	—[c]	—[c]
Hage et al.[15]	R-IB	1	5.3×10^5	—[c]	—[c]
	S-IB[d]	1	1.2×10^5	1	1.1×10^5

[a]The number of the binding sites for the high (n_1) and low (n_2) affinity sites.
[b]The binding constants for the high (K_1) and low (K_2) affinity sites.
[c]These values are not reported.
[d]Two types of high affinity sites are reported.

$K_2(R)$, and $K_2(S)$, respectively, where $K_1(R)$ and $K_1(S)$ are the binding constants of the high affinity site, and $K_2(R)$ and $K_2(S)$ are the binding constants of the low affinity sites for R-IB and S-IB, respectively.

The best fit model obtained using these parameter values is expressed with Eqs. 2 and 3.

$$r(R) = \frac{K_1(R) \cdot C_f(R)}{1 + K_1(R) \cdot C_f(R) + K_1(S) \cdot C_f(S)} + \frac{2 \cdot K_2(R) \cdot C_f(R)}{1 + K_2(R) \cdot C_f(R)} \quad (2)$$

$$r(S) = \frac{K_1(S) \cdot C_f(S)}{1 + K_1(S) \cdot C_f(S) + K_1(R) \cdot C_f(R)} + \frac{K_2(S) \cdot C_f(S)}{1 + K_2(S) \cdot C_f(S)} \quad (3)$$

where r(R) and r(S) are the number of the bound drug per albumin molecule for R-IB and S-IB, and $C_f(R)$ and $C_f(S)$ are the unbound concentrations of R-IB and S-IB, respectively. In the present model, R-IB and S-IB compete at the high affinity site, and the binding at the low affinity sites is independent of each other.

Using the present model, the unbound fraction of each enantiomer in the presence of various concentrations of the antipode were simulated, and the results were compared with the observed data. Calculated Fu(R) and Fu(S) values using Eqs. 2 and 3 are shown in Figure 4A and B, together with the observed values. Without assuming competitive interactions at the high affinity site, calculated Fu(R) and Fu(S) values in the presence of excess concentrations of the antipode were much smaller than those shown in Figure 4A and B.

Using Eqs. 2 and 3, binding of each enantiomer obtained with the racemate was simulated and compared with the observed data. In Figure 5A and B, Scatchard plots for R-IB and S-IB obtained with each enantiomer were compared to those obtained with the racemate. Plots obtained with the racemate were significantly different from those obtained with individual enantiomers (see Discussion). Solid lines in the figures represent simulated Scatchard plots of IB enantiomers in the presence of the antipode. Plots for both enantiomers obtained with the racemate were fairly well simulated using Eqs. 2 and 3, which supports the validity of the present model.

DISCUSSION

In the present study, the binding of IB enantiomers to HSA was measured in vitro using a chiral fluorescent derivatizing reagent. It was found that the binding of IB to HSA was stereoselective with the unbound fraction of S-IB being greater than that of R-IB. These observations are in agreement with the previous studies.[10–15]

Scatchard analyses revealed two classes of binding sites for both enantiomers. For the high affinity site the n value is approximately one for both enantiomers with the K value of R-IB being 2.3-fold greater than that of S-IB (Table 1). Both enantiomers were displaced from the high affinity site by diazepam, whereas phenylbutazone or digitoxin did not influence the binding of IB at the high affinity site. These results are consistent with the previous observations.[22–26] Therefore, it was confirmed that Site II is shared by IB enantiomers as the high affinity site.

Binding of IB to the low affinity sites appears to be in favor of R-IB, since the $n_2 \cdot K_2$ value of R-IB was approximately 3-fold greater than that of S-IB (Table 1). However, further studies are needed to clarify the stereoselectivity of the low affinity binding site. As shown in Figure 2, the Scatchard plots at higher concentrations ($r > 1$) are very close and almost parallel to the horizontal axis, which may result in incorrect estimation of the binding parameters of the low affinity sites. On the other hand, it was made clear that the low affinity sites of IB enantiomers were neither Sites I or III, since the binding of IB enantiomers at higher concentrations (2.5×10^{-4} M) was not influenced by phenylbutazone, diazepam, or digitoxin. Although diazepam displaced IB enantiomers to a small extent, the displacement was attributable to the displacement only at the high affinity site (data not shown).

Two classes of binding sites have been reported for IB when the binding was measured as the racemate. It has been reported that the binding constant (as the racemate) at the high affinity site is 1.76×10^5 M^{-1} by Lockwood et al.[27] and 7.93×10^5 M^{-1} by Montero et al.,[28] which are similar to those observed in the present study. However, the binding constant of 2.73×10^6 M^{-1} reported by Whitlam et al.[29] is greater than our values. All these studies were conducted at 37°C. The binding constant at the low affinity site is reported to be approximately 2×10^4 M^{-1} with the

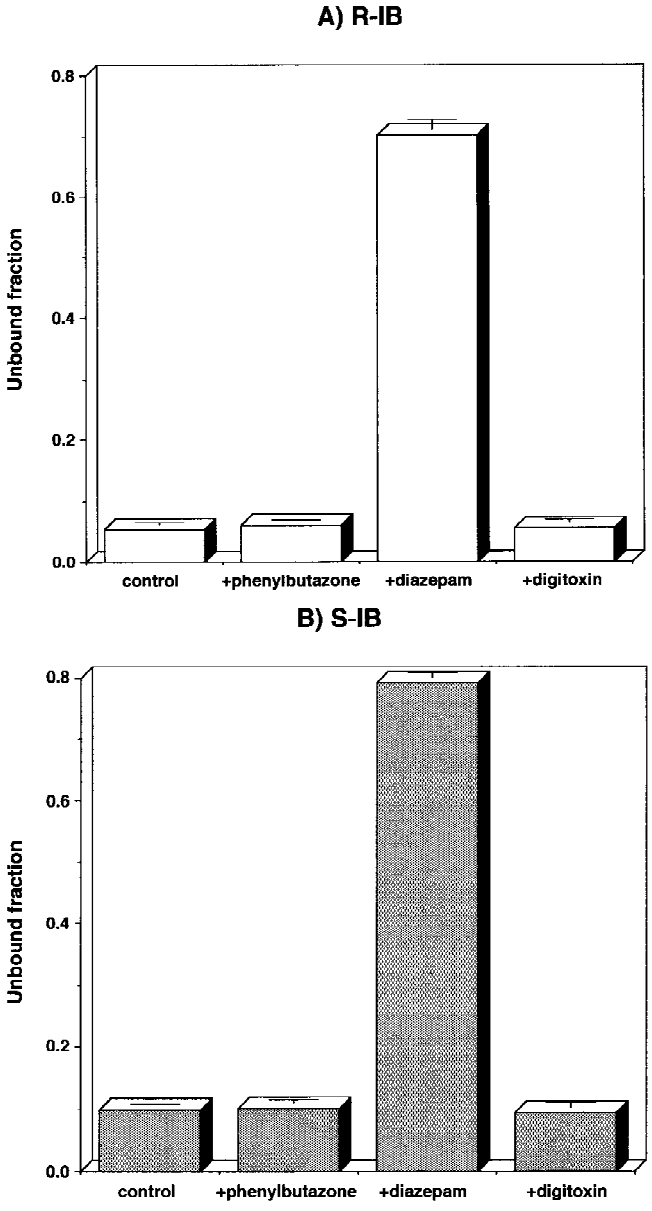

Fig. 3. Influence of phenylbutazone, diazepam, and digitoxin on the binding of R-IB **(A)** and S-IB **(B).** Concentration of each enantiomer was 2.5×10^{-5} M and the concentration of each site marker ligand was 3×10^{-4} M. Mean ± S.D., n = 3.

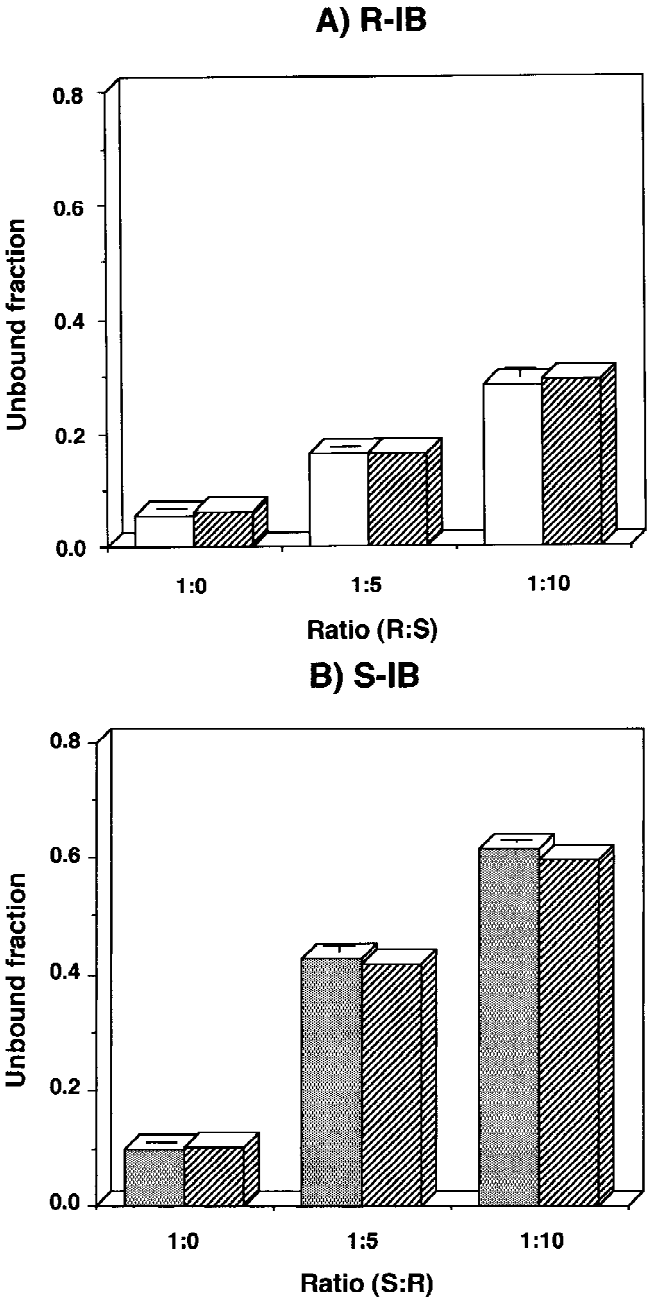

Fig. 4. Simulated and observed unbound fraction of R-IB **(A)** and S-IB **(B)** in 0.4% HSA at various R/S ratios. Concentration of each enantiomer was kept at 2.5×10^{-5} M, and the concentrations of the antipode were 0, 1.25×10^{-4}, and 2.5×10^{-4} M for the enantiomer:antipode ratio of 1:0, 1:5, and 1:10, respectively. Open and shaded columns represent the observed unbound fraction of R-IB and S-IB, respectively (mean ± S.D., n = 3). Hatched columns represent the simulated unbound fraction (see text for simulation).

number of the binding sites being 6–10.[28,29] The binding constants at the low affinity site appear to be similar to those obtained in the present study, whereas the number of the binding sites are different from our values.

With regard to the binding constants of IB enantiomers, several reported values are available (Table 2). Rahman et al.[13] measured the binding of IB enantiomers to HSA by equilibrium dialysis at 25°C. They used HPLC with UV detection for the determination of IB. Binding parameters obtained in their study are $n_1 = 1.4$, $K_1 = 3 \times 10^6$ M^{-1}, $n_2 = 4.7$, and $K_2 = 1.9 \times 10^5$ M^{-1} for R-IB and $n_1 = 1.2$, $K_1 = 2 \times 10^6$ M^{-1}, $n_2 = 4.5$, and $K_2 = 1.8 \times 10^5$ M^{-1} for S-IB. Paliwal et al.[14] measured the binding of IB enantiomers in human plasma obtained after in vivo administration. They used an ultrafiltration method at 37°C with ^{3}H-labeled IB enantiomers. They reported that the number of the binding sites is approximately one for both enantiomers and that the binding constants are 5.75×10^5 and 2.10×10^5 M^{-1} for R-IB and S-IB, respectively. They also reported competitive interactions of IB enantiomers. However, the existence of low affinity binding site was not reported in their study.

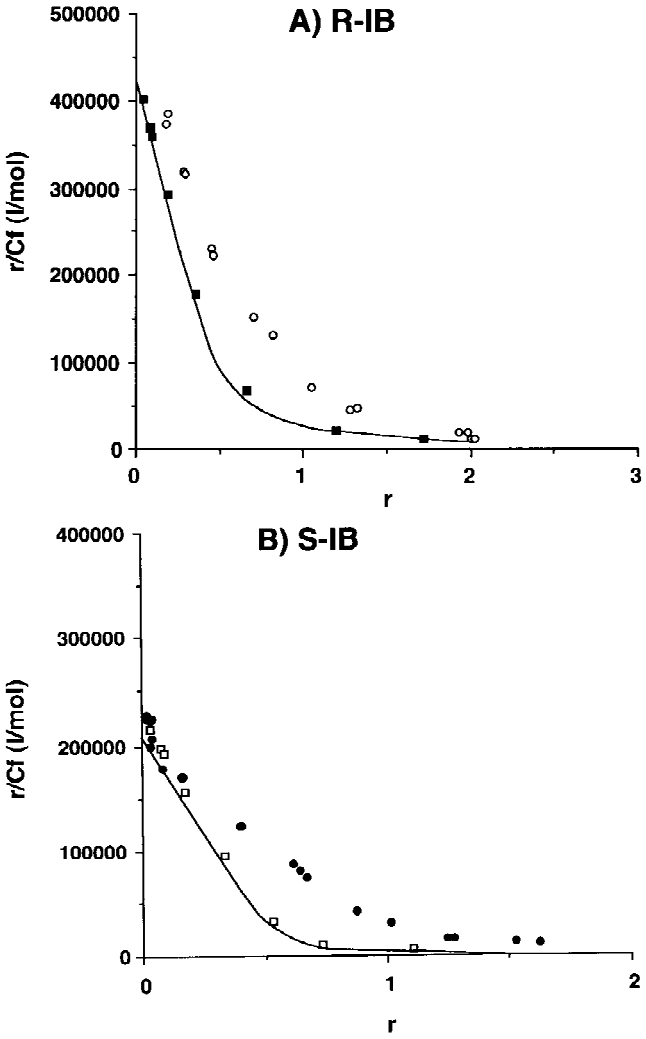

Fig. 5. Simulated and observed Scatchard plots for the binding of IB enantiomers in 0.4% HSA. ○: R-IB measured with R-IB alone; ●: S-IB measured with S-IB alone; ■: R-IB measured with the racemate; □: S-IB measured with the racemate; —: simulated curves (see text for simulation).

Finally, Hage et al.[15] measured the binding of IB enantiomers at 25°C using an HPLC column that contained immobilized HSA. According to their results, the number of the binding sites is one with the binding constant being 5.3×10^5 M^{-1} for R-IB. They also reported that two types of high affinity binding sites exist for S-IB with the binding constants being 1.1×10^5 and 1.2×10^5 M^{-1}, respectively.

The binding constants obtained in the present study are similar to those reported by Paliwal et al.[14] and Hage et al.[15] However, we observed only one binding site for the high affinity site of S-IB, which disagrees with the results obtained by Hage et al. The discrepancy may be due to the fact that immobilized HSA was used in their study. Immobilization of HSA may have affected the binding characteristics of the protein.

According to the present study, the binding constant of R-IB is 2.3-fold greater than that of S-IB. At therapeutic concentrations (approximately 10 μg/ml or 4.9×10^{-5} M) with the molar ratio of IB to HSA being approximately 0.1, stereoselectivity in the binding to HSA probably reflects that in the binding to the high affinity site.

The IB enantiomers appear to share Site II as the common high affinity binding site, which was reflected in significant enantiomer-enantiomer interactions at low IB concentrations (Fig. 4A and B). In order to study the interactions at the low affinity sites, unbound fraction was also measured at a constant concentration of higher concentration (2.5×10^{-4} M) in the presence of various concentrations (0–12.5×10^{-3} M) of the antipode. Both Fu(R) and Fu(S) increased with an increase of the concentration of the antipode, although the increase in the unbound fraction was less marked than that observed at a lower concentration (2.5×10^{-5} M) (data not shown). The results suggested that enantiomer-enantiomer interactions are much smaller at the low affinity binding sites.

Since the observations in the site marker ligand study strongly suggested that both enantiomers share a common binding site, the enantiomer-enantiomer interaction data were analyzed with the assumption of competitive interaction. As a result, the binding model described with Eqs. 2 and 3 predicted the interaction data best as shown in Figure 4A and B. According to the model, a single high affinity binding site (Site II) favors R-IB rather than S-IB, and IB enantiomers compete with each other at this site. Other sites are low affinity sites with the binding of the enantiomer being independent of the antipode. Using the present model, calculated Fu(R) and Fu(S) values at the high enantiomer concentration (2.5×10^{-4} M) in the presence of excess concentrations of the antipode were also in good agreement with the observed values (data not shown).

The validity of the present model was verified further by the good agreement between the calculated and observed Scatchard plots for the racemate (Fig. 5A and B). It should be noted that the slopes of the Scatchard plots for both enantiomers were greater for the racemate than those for R-IB or S-IB alone. When the racemate is used the concentration of the antipode is simultaneously increased with an increase of the enantiomer concentration, resulting in more marked displacement by the antipode. The observations indicate that it is not possible to obtain true binding parameters if binding is measured using a racemate.

A commercially available Fraction V HSA (Sigma) was also used to study the stereoselective binding of IB. The results obtained were similar to those observed in this study, suggesting that the binding characteristics of IB to HSA in vivo are similar to those observed in the present study. It is also supported by the fact that the present results are consistent with those obtained by Paliwal et al.[14] using human plasma.

In this study, binding of IB enantiomers to HSA was measured using a chiral fluorescent derivatizing reagent. Detailed analyses of the binding characteristics were made possible by using the present analytical method with high sensitivity and stereospecificity. Use of chiral fluorescent derivatizing reagents may help to clarify the pharmacokinetic characteristics of chiral drugs, especially in cases where a highly sensitive assay is needed.

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